REMARKS

Claims 1-14, 17 and 67-68 are pending in the present application. No amendments have been made and no new matter has been added.

CLAIM REJECTIONS

35 U.S.C. § 103

The Examiner has rejected claims 1-6, 17, 67 and 68 over Kallioniemi *et al.* U.S. Publication No. 2002/0132246 ("Kallioniemi") in light of McGill *et al.* U.S. Patent No. 5,658,730 ("McGill") on page 3, paragraph 6 of the Office Action.

The Examiner stated that Kallioniemi teaches the detection of gene copy number amplifications or deletions by hybridization of target nucleic acids to an array of a plurality of immobilized probes by the method of claim 1, except that Kallioniemi does not teach DNA fragments with a length of less than about 200 bp to less than about 30 bp.[1]

The Examiner alleged that McGill cures the deficiency of Kallioniemi.[2] The Examiner stated that McGill teaches the detection of chromosome 8 amplification using probes derived from the chromosome, wherein the probe lengths are 10-500 bp, and preferably, 20 bp.

Applicants respectfully traverse this rejection. Applicants submit that is no *prima facie* case of obviousness because there is no motivation to combine Kallioniemi and McGill. Further, Kallioniemi and McGill are not drawn to analogous art, and thus may not be combined in an obviousness rejection under 35 U.S.C. § 103. In addition, there are secondary considerations present here, including a solution to a long-felt but unsolved need and unexpected results.[3]

Secondary Considerations

There has been a long-felt but unsolved need for methods of array-based comparative genomic hybridization (CGI) which show decreased cross hybridization or repetitive and closely related sequences and thereby display improves the resolution of the resulting molecular profile of genomic DNA. Traditional methods of CGI use significantly longer labeled genomic fragments. In fact, many protocols recommend the use of long fragments to improve intensity

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[1] Office Action at page 5, paragraph "B".
[2] *Id.* at paragraph "C".

and uniformity of hybridization.[4] However, these longer fragments routinely result in repetitive sequence and other unwanted background cross hybridization on the immobilized nucleic acid. The methods of the present invention solves this long-felt need by providing target nucleic acids of less than 200bp which significantly reduces the amount of repetitive sequence hybridization and cross-hybridization from closely related sequences and significantly improves resolution.

Applicants' claimed methods require use of target labeled nucleic acids of less than 200 bp. The claimed methods achieve unexpectedly superior CGI resolution and reduction in the amount of repetitive sequence hybridization and cross-hybridization from closely related sequences.

As the specification teaches, when the size of the target labeled nucleic acid becomes less than 200 bp, the size of the unhybridized dangling ends decreases and the probability that the non-hybridized ends will further hybridize to another fragment of DNA, resulting in aggregating hybridization also decreases.[5] Aggregation hybridization makes hybridization less quantitative and also creates high background.[6]

Neither Kallioniemi, which does not mention target nucleic acids of less than 200 bp, nor McGill (which does not teach or suggest the use of target nucleic acids with CGH arrays), teach or suggest that these smaller nucleic acids would have such properties when used as probes in CGH arrays. Thus, the combination of Kallioniemi and McGill, could not lead the ordinarily skilled artisan to the solution to the long-felt need, nor to the unexpected and superior advantages (unexpectedly superior CGI resolution and reduction in the amount of repetitive sequence hybridization and cross-hybridization from closely related sequences) that the claimed invention provides.

Motivation to Combine

To establish a *prima facie* case of obviousness, it must be shown: first, that there is some suggestion or motivation, either in the reference or in the knowledge cited available to one of ordinary skill in the art, at the time the invention was made, to modify the reference to obtain the

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[3] *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966).

[4] Kallioniemi, Genes, Chromosomes & Cancer, 10:231-243, 1994. *See*, Specification at page 22, lines 26-30. Courtesy Copy Enclosed

[5] Instant specification at page 23, lines 1-8.

[6] *Id.*

invention and second, that the prior art reference teaches or suggests all the limitations of the claim.[7]

Kallioniemi and McGill do not describe the same technique for analyzing genomic sequences. Kallioniemi teaches methods of rapid molecular profiling using arrays.[8] McGill teaches methods of detecting prostate cancer by evaluating gene amplification in the q arm of chromosome 8 using fluorescent *in situ* hybridization of metaphase spreads.[9] One of ordinary skill in the art would not be motivated to use probes of the size shown in McGill to perform the array based assays in Kallioniemi.

Target nucleic acids of less than 200 bp are used in the array based methods of the invention because they significantly improve the resolution of the molecular profile analysis.[10] As discussed *supra*, the technique is particularly advantageous in array based comparative genome hybridization (CGH) because it lowers the possibility of partial hybridization to closely related sequences by probes under stringent conditions.[11] Moreover, aggregating hybridization is prevented through the use of smaller probes by the reduction of hybridization of dangling ends to closely homologous sequences.[12] Aggregating hybridization makes array-based CGH less quantitative and causes high background.[13]

Kallioniemi does not teach or suggest these problems of aggregating hybridization or high background. In fact, additional publications of Kallioniemi, which comprise the state of the art at the time of filing, recommend the use of long fragments to improve intensity and uniformity of hybridization.[14] McGill also does not teach or suggest problems of aggregating hybridization or high background, since these problems are only significant in array-based CGH.[15] McGill does not even mention array-based CGH. Because neither Kallioniemi nor McGill teach any underlying motivation for the use of smaller probes in array based CGH (and other publications of Kallioniemi teach away from smaller target nucleic acid fragments) one of

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[7] MPEP § 2143.
[8] *See* Kallioniemi at the title and abstract.
[9] *See* McGill at the Figures.
[10] *See* the instant specification at page 8, lines 21-23.
[11] *Id.* at page 9, lines 9-13.
[12] *Id.* at page 9, lines 15-22.
[13] *Id.* at page 9, lines 19-24.
[14] Kallioniemi, Genes, Chromosomes & Cancer, 10:231-243, 1994. *See*, Specification at page 22, lines 26-30. Courtesy Copy Enclosed

ordinary skill in the art would not have been motivated to combine the teachings of Kallioniemi with the teachings of McGill to reach the present invention.

Non-analogous art

In order for a reference to be used in a rejection under 35 U.S.C. § 103, it must be properly contained within the scope of the relevant prior art.[16] There is a two step test for determining whether a reference is analogous art.[17] First, it must be ascertained whether the reference is within the field of the inventor's endeavor.[18] Second, if the answer to the first question is no, it must be ascertained whether the reference is reasonably pertinent to the particular problem the inventor was involved in.[19] If the answer to both of the above questions is no, then the art is nonanalogous, and not properly contained within the scope of the prior art.[20] Applicants assert that McGill is nonanalogous art and thus is not available to the Examiner for use in a claim rejection under 35 U.S.C. § 103.

McGill is not in the same field of endeavor as the invention of claims 1-6, 17, 67 and 68. Both McGill and the invention are drawn to comparative genomic hybridization (CGH) but not to the same method of applying this technology. Whereas McGill teaches fluorescent *in situ* hybridization of metaphase spreads, the instant claims are drawn to a plurality of DNA molecules on an array. Simply because a reference is considered to be in the same industry as an invention does not necessarily mean that the reference is analogous art.[21]

In *In re Clay,* the Federal Circuit ruled that a gel used in an underground natural oil-bearing formation was not in the same field of endeavor as a gel used to fill the dead space in a man-made storage tank.[22] The court reasoned that differences in temperature and pressure in the oil well and storage tank, and the nature of the well and the tank led the court to rule that the inventors of the gel used in the oil well were in the field of endeavor of extracting oil, while the

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[15] *Id.* at page 9, lines 9-13.
[16] *In re Deminski* 796 F.2d 436, 441 (Fed. Cir. 1986).
[17] *Id.* at 442.
[18] *Id.*
[19] *Id.*
[20] *Id.* at 441. Also *see* MPEP § 2141.01(a).
[21] *In re Clay*, 966 F.2d 656, 659 (Fed. Cir. 1992).
[22] *Id.*

inventors of the gel used in the storage tank were concerned with the field of invention of storing oil.[23]

Likewise, while both the invention of claims 1-6, 17, 67 and 68 and the teachings of McGill are drawn to CGH, because of the different methods involved, they are not in the same field of endeavor. The instant invention is in the field of endeavor of generating a molecular profile of genomic DNA by hybridization of a genomic DNA target to a plurality of immobilized nucleic acid probes, wherein the plurality is a collection of clones that represent all of a chromosome or a genome of an organism. The field of endeavor for the inventors in McGill was comparing the amplification of one gene at one arm of one chromosome. The array based CGH of the instant invention is the best means for generating the representation of a genome of an organism while the FISH based metaphase spreads of McGill was the best means for comparing one gene at one site. Thus, McGill is based in a distinct field of endeavor, the detection of amplification or deletion of one gene at one locus, than the methods of the instant claims, the analysis of multiple diverse genetic changes in a genome simultaneously.

Moreover, McGill is not reasonably pertinent to the particular problem of the invention of claims 1-6, 17, 67 and 68. McGill teaches the use of probes of 200 bp or fewer in FISH based metaphase spreads, but does not teach their use in array based CGH. The problems of aggregating hybridization and heightened background are not present in the same ways in FISH based metaphase spreads as they are in array based CGH. Thus, the teachings of McGill are not reasonably pertinent to the problem of claims 1-6, 17, 67 and 68.

Thus, because McGill is not in the same field of endeavor as the invention of claims 1-6, 17, 67 and 68 and is not reasonably pertinent to the particular problem of the invention of claims 1-6, 17, 67 and 68, it is not analogous art and cannot be used as prior art for a rejection under 35 U.S.C. § 103.

Rejections in light of Anderson and Ordahl

The Examiner has also rejected claims 7, 8 and 10 over Kallioniemi and McGill in light of Anderson Nucl. Acids Res., 9:3015-27 ("Anderson") on page 5, paragraph 7 of the Office Action. As explained above, Applicants assert that the instant specification sets forth unexpected

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[23] *Id.*

and superior properties not taught by Kallioniemi and McGill. Further, there is no motivation to combine Kallioniemi and McGill. Additionally, McGill is not drawn to analogous art to the invention of claims 7, 8 and 10, and thus may not be combined in a rejection of these claims under 35 U.S.C. § 103.

Moreover, one of ordinary skill in the art would not have a motivation to combine Anderson with Kallioniemi and McGill. Anderson teaches sequencing methods. It does not teach CGH arrays or methods of using them. It is irrelevant whether Anderson teaches fragmentation of genomic DNA to sizes of less than 200 bp or not, because it does not teach any advantage of using nucleic acid molecules under 200 bp in length in CGH arrays. Kallioniemi and McGill do not cure the deficiencies of Anderson. There is no suggestion in Kallioniemi, McGill or Anderson to combine these references to arrive at the invention of claims 7, 8 and 10. For all of the above reasons, Applicants assert that claims 7, 8 and 10 are non-obvious over the combination of Kallioniemi, McGill and Anderson and request that this rejection be withdrawn.

The Examiner has also rejected claim 9 over Kallioniemi and McGill in light of Waggoner *et al.* U.S. Patent No. 5,268,486 ("Waggoner") on page 6, paragraph 8 of the Office Action. As explained above, Applicants assert that the instant specification sets forth unexpected properties not taught by Kallioniemi and McGill. Further, there is no motivation to combine Kallioniemi and McGill. Additionally, McGill is not drawn to analogous art to the invention of claim 9, and thus may not be combined in a rejection of these claims under 35 U.S.C. § 103. Waggoner does not cure the deficiencies of Kallioniemi and McGill because it does not teach CGH arrays or any of the advantages of the use of the target nucleic acid fragments of the invention with such arrays. Therefore, Applicants request that this rejection be withdrawn.

The Examiner has also rejected claim 11 over Kallioniemi, McGill and Anderson in light of Ordahl *et al.* Nucl. Acids Res. 3:2985-2999 (1976) ("Ordahl") on page 7, paragraph 9 of the Office Action. As explained above, Applicants assert that the instant specification sets forth unexpected properties not taught by Kallioniemi and McGill. Further, there is no motivation to combine Kallioniemi, McGill or Anderson. Additionally, McGill is not drawn to analogous art to the invention of claim 11, and thus may not be combined in a rejection of these claims under 35 U.S.C. § 103.

The Examiner alleged that Ordahl provides the motivation to combine the above references in the first paragraph on page 2985 of Ordahl. Ordahl does teach that using DNA fragments smaller than 500 bp in length permits the separation of repetitive and non-repetitive components of most eukaryotic genomes, but it does not teach the arrays of claim 11, nor does it teach that fragments of less than 200 bp are advantageous. The fragments of Ordahl have a mean length of 220-235 bp.[24] Claim 11 depends from claim 1, which stipulates that each labeled fragment consists of a length smaller than about 200 bases. Moreover, there is no motivation in Ordahl to further fragment the DNA probes with DNase. According to Ordahl, the fragments need only be smaller than 500 bp.[25] These fragments are produced with shearing without the use of DNase. Thus, Applicants submit that there is no motivation to combine Ordahl with the teachings of Kallioniemi, McGill and Anderson to reach the present invention. For all the above reasons, Applicants request that this rejection be withdrawn.

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[24] Ordahl at first paragraph of page 2986.
[25] *Id.*

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicants respectfully submit that the pending claims are in condition for allowance.

If the Examiner has any questions regarding these amendments and remarks, the Examiner is encouraged and invited to contact the undersigned at the telephone number provided below.

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GENES, CHROMOSOMES & CANCER 10:231–243 (1994)

Optimizing Comparative Genomic Hybridization for Analysis of DNA Sequence Copy Number Changes in Solid Tumors

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Comparative genomic hybridization (CGH) is a powerful new method for molecular cytogenetic analysis of cancer. In a single hybridization, CGH provides an overview of DNA sequence copy number changes (losses, deletions, gains, amplifications) in a tumor specimen and maps these changes on normal chromosomes. CGH is based on the in situ hybridization of differentially labeled *total genomic tumor DNA* and normal reference DNA to *normal* human metaphase chromosomes. After hybridization and fluorescent staining of the bound DNAs, copy number variations among the different sequences in the tumor DNA are detected by measuring the tumor/normal fluorescence intensity ratio for each locus in the target metaphase chromosomes. CGH is in particular useful for analysis of DNA sequence copy number changes in common solid tumors where high-quality metaphase preparations are often difficult to make, and where complex karyotypes with numerous markers, double minutes, and homogeneously stained chromosomal regions are common. CGH only detects changes that are present in a substantial proportion of tumor cells (i.e., clonal aberrations). It does not reveal translocations, inversions, and other aberrations that do not change copy number. At present, CGH is a research tool that complements previous methods for genetic analysis. CGH will advance our understanding of the genetic progression of cancer and highlight important genomic regions for further study. Direct clinical applications of CGH are possible, but will require further development and validation of the technique. We describe here our recent optimized procedures for CGH, including DNA labeling, hybridization, fluorescence microscopy, digital image analysis, data interpretation, and quality control, emphasizing those steps that are most critical. We will also assess sensitivity and resolution limits of CGH as well as discuss possible future technical improvements. *Genes Chromosom Cancer 10:231–243 (1994).* © 1994 Wiley-Liss, Inc.

INTRODUCTION

Development of a technique for genome-scale DNA sequence copy number analysis was motivated by the expectation that improved detection of DNA gains, amplifications, losses, and deletions would be valuable in cancer research and clinical diagnostics (Kallioniemi et al., 1992). Despite the recent advances in classical and molecular cytogenetics, relatively little is known on cytogenetic aberrations in common solid tumors compared with the enormous database of detailed information on changes in hematological malignancies (Mitelman, 1991). Few of the genes implicated in the development of these tumors have been identified. For example, several studies suggest that chromosomal structures indicative of gene amplification, i.e., homogeneously staining regions (hsr) and double minute chromosomes (dmin), are frequently found in breast and colorectal cancer, but often do not contain known oncogenes (Bruderlein et al., 1990; Saint-Ruf et al., 1990; Zafrani et al., 1992). Identification of the chromosomal origin of such amplified sequences would highlight locations of potentially important new oncogenes (Schwab and Amler, 1990; Kallioniemi et al., 1993a). Similarly, genome-scale allelic loss studies (allelotyping) for recognition of potential tumor suppressor gene loci in solid tumors are very labor-intensive (Vogelstein et al., 1989; Devilee et al., 1991). If physical deletions played a major role in the generation of allelic loss, a genome-wide DNA sequence copy number analysis would provide information on all potential tumor suppressor loci in a single experiment.

The first genomic in situ hybridizations were based on a single color strategy hybridizing labeled DNA from cancer cell lines to normal metaphase chromosomes. Unlabeled normal genomic DNA and Cot-1 were used to suppress the binding of labeled DNA sequences. Hybridizations with

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DNA from cancer cell lines that were known to have high-level amplifications of oncogenes (e.g., *MYC* amplification in COLO320HSR and *MYC* and *ERBB2* in SK-BR-3) resulted in strong hybridization signals on normal metaphase chromosomes at loci that corresponded to the known locations of these genes (8q24 for *MYC*, 17q12 for *ERBB2*). These early experiments also showed evidence of high-level amplifications at several chromosomal loci that were not previously known to give rise to DNA amplification or contain oncogenes. Subsequently, single-color hybridizations of labeled genomic DNAs to normal metaphase chromosomes have also been used by Joos et al. (1993) to find amplified sequences in tumors.

While single color hybridizations were adequate for detection of large copy number changes associated with high-level amplifications, variability in the intensity of the hybridization signal at different locations within a metaphase spread, and between different spreads, made it difficult to detect smaller copy number changes accurately across the genome. The inclusion of normal genomic DNA, detected with a different color than the tumor DNA, provided an internal hybridization intensity control at each location in the genome. This dual color hybridization permitted recognition of smaller copy number differences, including deletions. We called the technique comparative genomic hybridization (CGH) to emphasize the presence of two (or potentially more) differentially labeled genomic probes in the hybridization (Kallioniemi et al., 1992) (Fig. 1). Comparisons between multiple abnormal genomes are possible, but since the predominant practice involves comparison of tumor DNA with a normal reference, we will limit our discussion to measurements of this type. In this form of CGH, the primary data are profiles of the ratio of the intensities of the two fluorochromes along all target metaphase chromosomes (Kallioniemi et al., 1992; Piper et al., 1994). This "copy-number-karyotype" is a quantitative display of the variation in DNA copy number throughout the tumor genome (Fig. 2).

CGH has now been applied to the analysis of genetic changes in a number of different cancer cell lines, as well as primary breast, bladder, and ovarian tumors (duManoir et al. 1993; Kallioniemi et al., 1993b;1994; Sakamoto et al., 1993). In these experiments, CGH has already fulfilled the expectations. For example, over 20 different loci undergoing amplification (Kallioniemi et al., 1994) have now been mapped in breast cancer, most of these at chromosomal loci that were not previously known to undergo amplification or contain activated oncogenes. Similarly, genome-wide mapping of deleted regions in primary tumors has now become possible.

DETAILS OF THE CGH ANALYSIS AND THE CRITICAL STEPS OF THE PROCEDURE

While CGH has turned out to be highly useful, experience has also shown that establishment and routine application of the technique is demanding and requires fine tuning of methods for each step in the protocol: (1) preparation of normal metaphase spreads that serve as targets for the hybridization, (2) isolation of high-molecular-weight genomic DNA from tumor specimens, (3) labeling of the tumor and normal DNA with different haptens, (4) in situ hybridization of the labeled DNAs to a normal metaphase, washing the unbound DNA fragments and fluorescent staining of the bound ones, (5) fluorescence microscopy to visualize color ratio differences along metaphase chromosomes, (6) acquisition and display of multi-color digital images, (7) quantitation of copy number differences by generating green to red fluorescence intensity ratio profiles for all chromosomes in a metaphase spread, (8) combining the profiles from several metaphases to improve signal to noise ratio, and (9) interpretation of ratio changes and verification of results by control experiments.

We have previously published a CGH protocol used in early experiments (Kallioniemi et al., 1992). However, the technique has recently been refined to make the analysis more accurate and to facilitate studies of large numbers of primary tumors. Since our first publication, other laboratories have published their CGH protocols that differ somewhat from ours (duManoir et al., 1993; Joos et al., 1993). This article is intended to give a more detailed picture of our current practice of CGH, how it has evolved and how it is likely to develop further. We will base our discussion on the most recent optimized protocol for CGH (see appendix) as well as on our previously published descriptions of CGH (Kallioniemi et al., 1992) and fluorescence in situ hybridization (FISH) (Pinkel et al., 1988). We will emphasize the areas that are most critical and give guidelines as to how CGH data should be interpreted and validated, and how the quality of the procedure should be assessed.

Metaphase Chromosome Preparations for CGH

Normal metaphase chromosomes serve as hybridization targets for CGH. The quality of the CGH analysis is extremely dependent on the char-

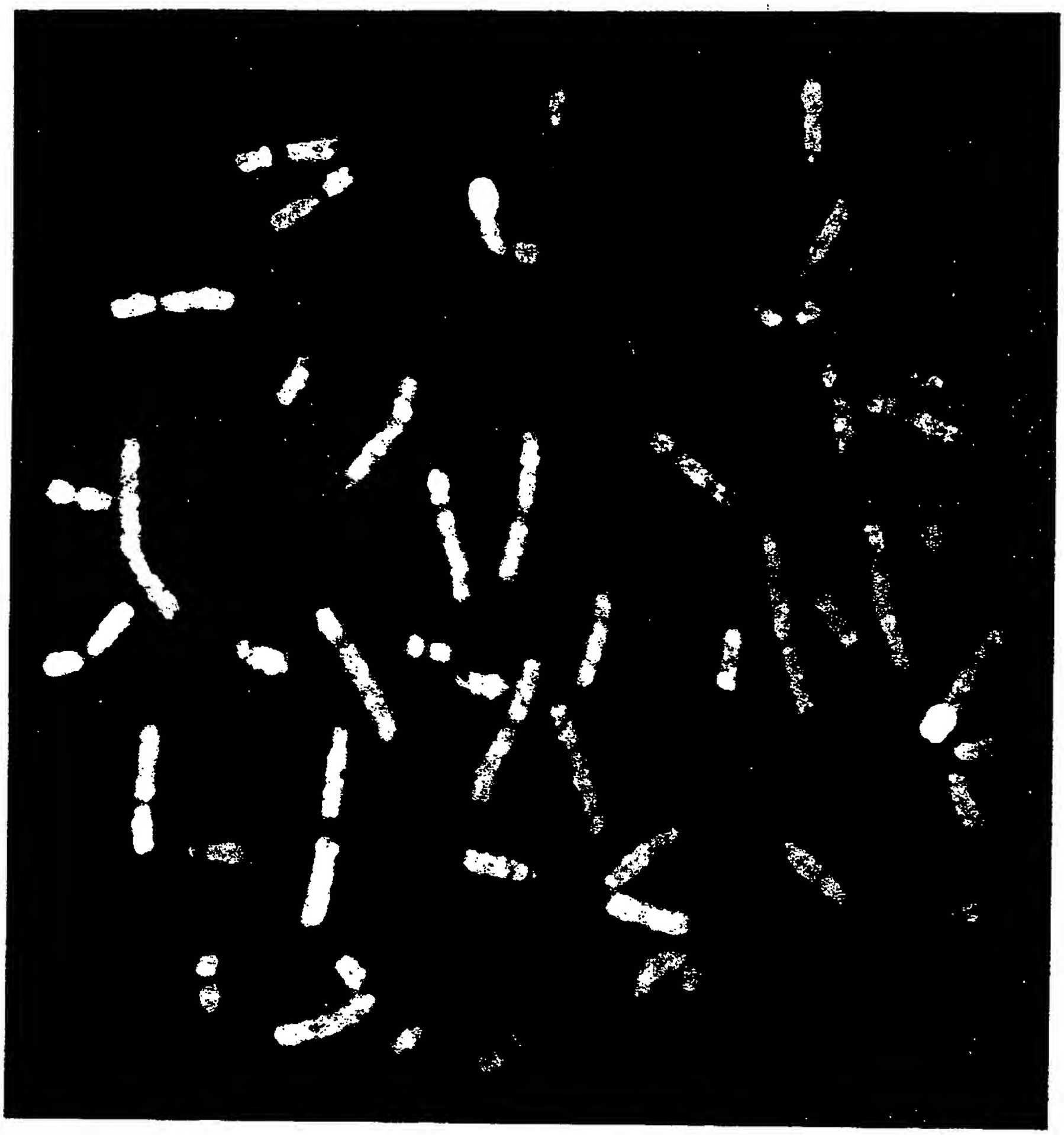

Figure 1. An example of a digital image of a CGH experiment. DNA from the ZR-75-30 breast cancer cell line (labeled in green) and normal reference DNA (labeled in red) were cohybridized with unlabeled Cot-1 DNA to a normal peripheral blood metaphase spread. Chromosomal regions that were over-represented (amplified) in the tumor are visualized in a predominantly green color, whereas regions that were lost (deleted) from the tumor are seen as regions with a predominantly red color. The chromosomes were counterstained with DAPI (blue color). For this figure, three different exposures of the same metaphase were made (matching DAPI, FITC, and rhodamine stains), the images were overlaid in pseudo-colors, color-balanced and contrast-stretched for display of the color differences.

acteristics of the metaphase spreads. Presumably this is because each element of the probe is present at a very low concentration so that accessibility of the target sequences to the probe is critical. Slides that are adequate for whole chromosome probe or cosmid hybridizations do not necessarily give good results in CGH.

We prepare prometaphase chromosome spreads for CGH according to routine procedures from PHA-stimulated, methotrexate-synchronized peripheral blood lymphocytes. A large number of slides are prepared at once, so that an entire experiment can be carried out with one batch of slides. The hybridization characteristics of all batches of slides are tested using labeled DNAs from normal cells and from a control cell line. De-

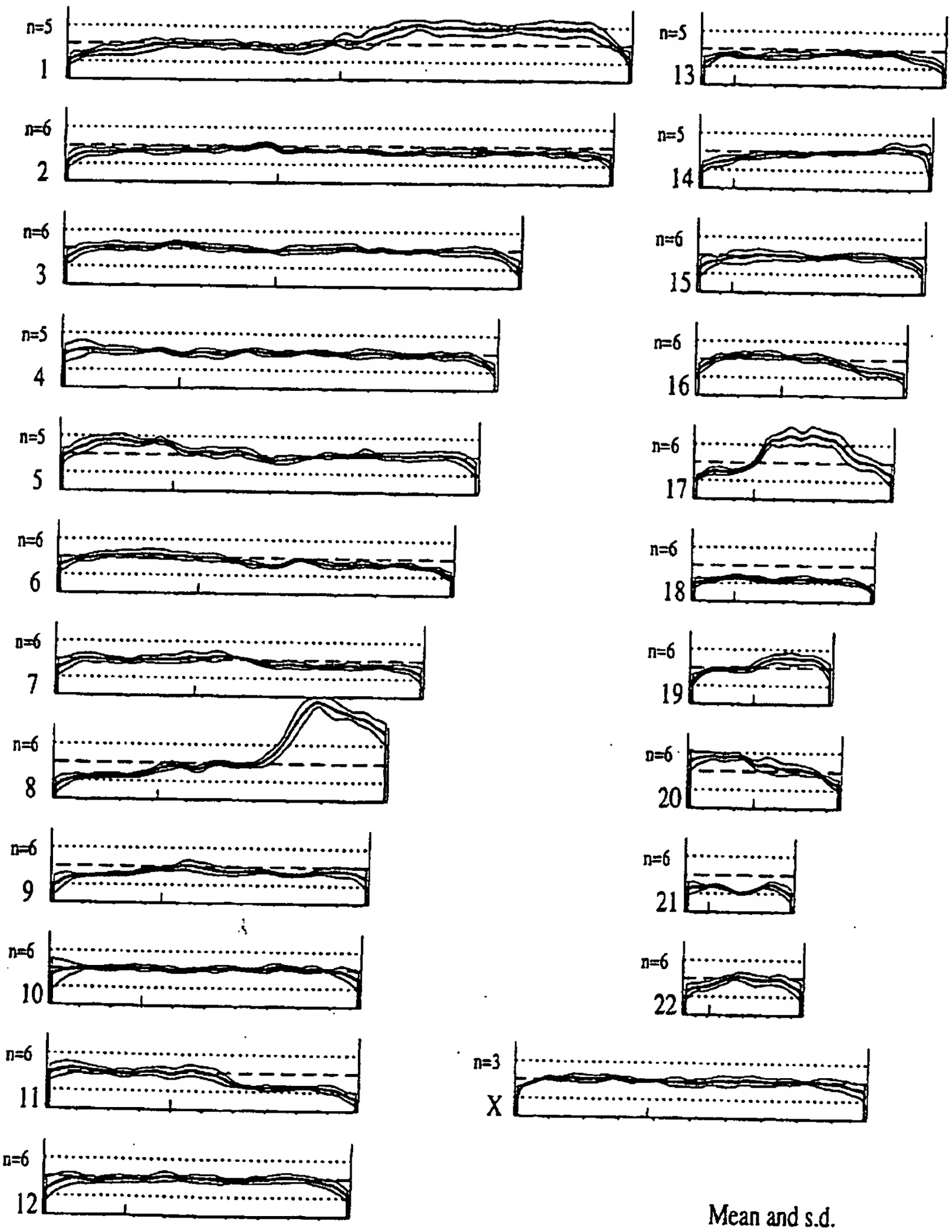

Figure 2. Quantitative digital image analysis of fluorescence intensity ratios. Green to red fluorescence intensity ratio profiles are shown for all chromosomes ("copy number karyotype") in the ZR-75-30 breast cancer cell line (see Fig. 1). The mean ratio (thick line) and ± 1 S.D. (thin lines) of six measurements are shown. The ratio profiles for each chromosome are shown from pter to qter (left to right). The baseline value (1.0) representing the mean green to red ratio for the entire metaphase is shown in a dashed line and ratios 0.5 and 1.5 as dotted lines. Based on this analysis, the chromosomal regions that were over-represented in the ZR-75-30 cell line were 1q, 5p, 8q23-q24, 17q12-q24, 19q, and 20p, whereas 8p, 9p, 11q13-qter, 16q23-qter, 17p, 18, and 21 were deleted.

Mean and s.d.

spite the optimization of denaturation and slide pre-treatment conditions for each batch of slides (see "Hybridization and Staining for CGH"), high-quality hybridization often cannot be achieved. In this case, the entire batch is abandoned. The reasons for the variable hybridization characteristics usually remain unknown. For example, deficient preparations are often morphologically indistinguishable from those that hybridize well.

In CGH analysis, all reasonably straight, non-

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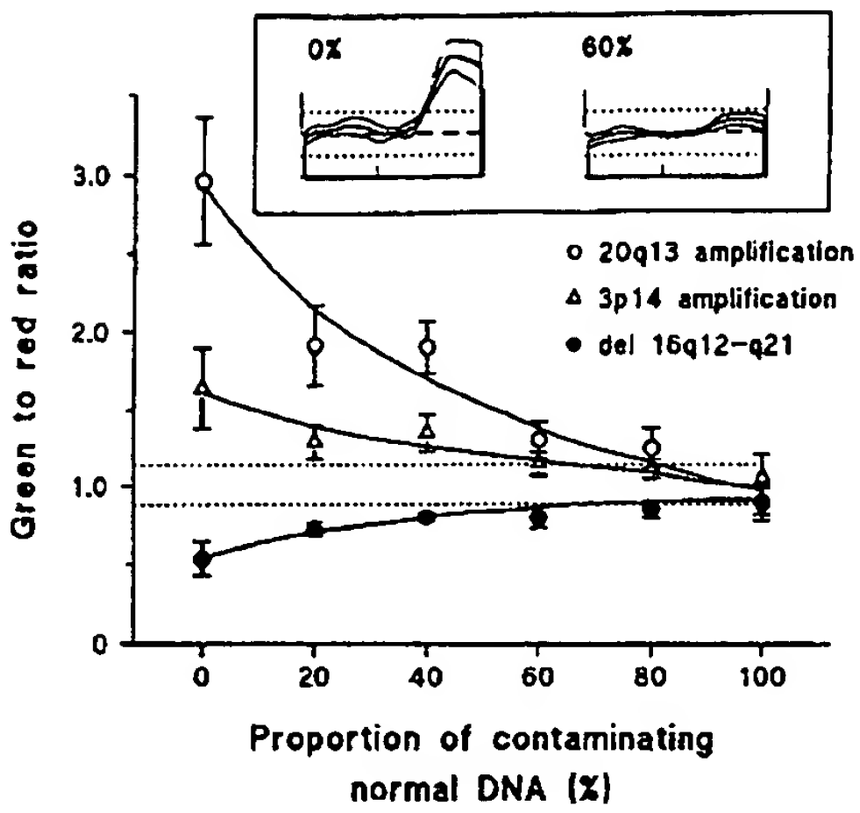

Figure 3. The effect of contaminating normal cells within a tumor specimen on the green to red fluorescence ratio. The measurement with 0% contaminating DNA reflects a hybridization with MCF-7 breast cancer cell line DNA (labeled in green) against a normal reference DNA (in red). In other hybridizations, an increasing concentration of normal DNA was mixed with the MCF-7 cell line DNA prior to DNA labeling. With increasing proportion of contaminating (green-labeled) normal DNA in the hybridization, the magnitude of the color ratio changes becomes smaller and more difficult to detect. The graph depicts the maximum green to red ratio deviation for three different aberrations present in the MCF-7 cell line (20q13 and 3p14 amplifications and 16q12-q21 deletion). Detection limits for ratio changes (0.85–1.15) determined based on normal vs. normal hybridizations are shown with dotted lines. The insert shows the original ratio profile for chromosome 20 and the ratio profile with 60% contaminating normal cells.

overlapping chromosomes are analyzed from each metaphase and data from several spreads are combined. Thus, the procedure is more efficient if the number of overlapping chromosomes is minimal. Preparations with too short chromosomes are not analyzed as the resolution along the chromosome axis will be poor. We currently use DAPI as the counter-stain to obtain a faint R-banding pattern along the chromosomes. Besides adequate hybridization quality, the chromosomes also need to retain their morphology so that they can be identified and hybridization intensity changes assigned to chromosome bands.

Tumor Specimens and DNA Isolation

It is of paramount importance to ascertain that DNA is isolated from specimens that are histologically representative of the tumor and contain as high a proportion of malignant cells as possible. Contaminating normal cells significantly dilute the ability to see copy number changes (Fig. 3). Necrotic tissue and regions with inflammation may contain degraded DNA and many normal cells and should be avoided.

Most methods for extracting DNA from cell lines and primary tumors yield high-molecular-weight DNA suitable for CGH. Relatively high-molecular weight DNA can often also be obtained from formalin-fixed paraffin-embedded tissues. However, when labeled and used for CGH, the quality of the hybridization is often poor and does not meet our requirements for reliable interpretation of copy-number karyotype (see "Criteria for an Acceptable CGH Image"). However, improvement of the technology is under way and may lead to much better quality of CGH from archival tissue (Speicher et al., 1993; Isola et al., unpublished observations).

DNA Labeling for CGH

We currently use nick translation for labeling genomic DNAs with biotin-14-dATP (tumor DNA) and digoxigenin-11-dUTP (normal DNA). Directly fluorochrome-conjugated nucleotides, such as FITC-dUTP and Texas Red-dUTP can also be used (see appendix for a protocol). Compared to labeling of centromeric and cosmid probes, the optimal fragment length of genomic probes after nick translation is distinctly longer (a smear from 600 to around 2,000 bp in a double stranded agarose gel). The fragment length is modified by adjusting the ratio of DNase to DNA polymerase in the nick translation reaction. At the probe concentrations used in our hybridizations, these long fragments dramatically improve the intensity and uniformity of the hybridization as compared to 300–600 bp fragments usually obtained with nick translation kits. Other techniques, such as random priming (unpublished data) and degenerate-primer PCR (Speicher et al., 1993) may also be useful for labeling genomic DNA.

Hybridization and Staining for CGH

Hybridization and staining protocols essentially follow those used in chromosome painting and FISH with cosmid probes (Pinkel et al., 1988; Kallioniemi et al., 1992). We currently use 120 ng (biotin and digoxigenin-labeled DNAs) to 200 ng (direct fluorochrome-conjugated DNAs) of each of the labeled DNAs per hybridization. Five to 10 μg of Cot-1 DNA is added to the hybridization to block binding of the labeled repetitive sequences in both genomes. Repeat sequences, if not adequately blocked, tend to result in large ratio changes at the peri-centromeric and heterochromatic regions. To achieve the required DNA concentration, the labeled DNAs and blocking DNA are mixed, ethanol-precipitated, and dissolved in the hybridization buffer.

Before hybridization, the slides are denatured at

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72–74°C for 3 minutes in 70% formamide/2 × SSC, followed by a proteinase K digestion. Optimal denaturation time and temperature, as well as the amount of proteinase K required, varies from one batch of slides to another. The aim is to treat the slides as harshly as possible to maximize denaturation and probe penetration, but avoid destroying the DAPI banding pattern that is necessary for chromosome identification. Hybridization is carried out under a coverslip in a moist chamber at +37°C for 2 days.

Washing of unbound probes is carried out as in regular FISH protocols (Pinkel et al., 1988; Kallioniemi et al., 1992). If indirectly labeled probes are used, immunochemical staining with avidin-FITC (providing green fluorescence for tumor DNA) and anti-digoxigenin rhodamine (red fluorescence for normal DNA) is carried out. Only one layer of each detection reagent is used since this results in the highest signal to noise ratio and the most uniform signal.

Fluorescence Microscopy and Visual Analysis of CGH

CGH analysis is dependent on the evaluation of the relative intensities of the two fluorochromes. This can be accomplished either by visual multicolor fluorescence microscopy or computer-assisted multicolor image analysis. For visual analysis, the intensity of two fluorescent dyes must be compared along the length of a chromosome. This requires the use of double or triple band pass filters that allow simultaneous visualization of two (e.g., FITC and rhodamine) or more fluorophores. High-level gene amplifications (> 10–20-fold) as well as changes affecting large regions, such as gains and losses of entire chromosome arms, are visually detectable in homogeneous cell lines. Because of the limited capability of the human eye in detecting small variations in color balance, small deletions and other lower level copy number differences often cannot be reliably detected. Ratio changes found in the analysis of primary tumors characterized by genetic heterogeneity and contaminating normal cells (see Fig. 3) often cannot be reliably evaluated without digital imaging.

Acquisition and Display of MultiColor Digital Images

The use of digital image analysis improves both the quantitation and display of CGH results. Our analysis procedure is based on both the visual evaluation of contrast-stretched digital images and quantitative analysis of fluorescence ratios.

The acquisition of images is carried out by first carefully scanning through the entire slide to find the best metaphase cells. Routinely, images of 5 or more metaphases of the highest quality are collected from each slide. Images are acquired using a multicolor quantitative image processing system (QUIPS) developed at the UCSF Division of Molecular Cytometry (manuscript in preparation). QUIPS is based on a regular fluorescence microscope (Nikon SA and Zeiss Axiophot have been used) equipped with a high-resolution cooled (Photometrics Inc., Tucson, AZ) or non-cooled CCD camera (Xillix Inc., Vancouver, B.C.), and triple band pass beam splitter and emission filters (P-1 filter set, Chroma Technology). Gray level images of each of the fluorochromes are obtained using a computer-controlled filter-wheel to insert single-band-pass excitation filters to sequentially excite DAPI, FITC, and rhodamine. This allows visualization of all three fluorochromes without any registration shifts between images and with negligible cross-talk between the fluorochromes. Exposure times for each color are adjusted to obtain high intensity without saturation.

As CGH requires acquisition of high-resolution images from the entire metaphase spread, the resulting images can be composed of up to 1 million pixels (1,000 by 1,000 picture elements). The three fluorochrome-specific images obtained from each metaphase with the CCD camera are stored in a Sun workstation for subsequent analysis. For visual assessment, the three single-color images are overlaid and displayed in pseudo-colors matching approximately the original colors of the fluorochromes and counterstain used (Fig. 1). Software developed based on the Scilimage package (TNO, Delft, Netherlands) is used for the acquisition and display of multicolor images. By display adjustments, the relative intensities of green and red fluorescence are balanced and small color ratio differences made more readily visible through the use of contrast-stretching. Chromosomes are identified based on a contrast-stretched image of the DAPI counterstain.

Criteria for an Acceptable CGH Image

The capabilities of CGH in detecting copy-number differences are critically dependent on the quality of the hybridization. Sophisticated image analysis, combining data from numerous metaphases, cannot compensate for poor hybridization quality. We first visually inspect all images collected to ascertain that the quality is adequate

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for quantitative ratio profile analysis. The following criteria are used:

1. All metaphase spreads show smooth high-intensity hybridization. Granularity is the most common problem leading to increased noise. Metaphases with non-specific granules and fluorescent spots on the chromosomes are not analyzed. Analysis of metaphases where the hybridization intensities of both colors along chromosomes show a prominent variation that resembles a banding pattern may be unreliable. This may be due to inadequate denaturation of target DNA.
2. Green and red fluorescence distributions are similar between the two sister chromatids of a chromosome, the two chromosome homologues in each metaphase and the same chromosomes in different metaphases.
3. Background fluorescence level on the slide surrounding the chromosomes is low and uniform.
4. Binding of the labeled DNAs to chromosome centromeres and heterochromatic regions is low.
5. Chromosomes show intense DAPI staining with visible bands, adequate length, and minimal overlapping.

Quantitative Analysis of Green and Red Fluorescence Ratio Profiles for CGH

Quantitative analysis of green to red fluorescence ratios along metaphase chromosomes gives the most objective assessment of copy number changes. Quantitative ratio analysis is done using the Xwoolz program which is based on chromosome image analysis algorithms developed at the MRC Human Genetics Unit, Edinburgh, for computer-assisted karyotyping of G-banded metaphases (see Piper et al., 1994, for a more detailed description).

The chromosomes are first segmented based on the combined DAPI and red (reference DNA) images. In order to be able to compare ratios between different metaphases, and between different slides or hybridizations, it is necessary to standardize the green and red fluorescence intensities of each metaphase so that the effects of differences in the hybridization and imaging conditions (such as concentration and labeling intensity of the two DNAs, camera integration time, bleaching, etc.) between each experiment are minimized. Scaling factors are computed using global background subtraction to normalize the green and red fluorescence intensities so that the average normalized green to red intensity ratio is 1.0 for the entire metaphase. For this purpose, we have found that variation in the background level across the image can be ignored (Piper et al., 1994).

Next, minor errors in the segmentation of chromosomes are corrected interactively, for example touching chromosomes separated. Then the operator selects the chromosomes (previously identified visually based on DAPI banding) one at a time from the metaphase. For each chromosome, the software measures the background intensities of each fluorochrome in the immediate vicinity of the chromosome. This background level is defined as the fall set boundary, a set of pixels surrounding the chromosome of interest, along a path of strictly decreasing fluorescence intensity (see Piper et al., 1994). The medial or symmetry axis of the chromosome is then determined and displayed. Green and red fluorescence intensities, corrected by subtracting the local background and then multiplied by the intensity scaling factors found earlier, are integrated along lines that are perpendicular to the medial axis and spaced at one pixel intervals along the axis. The integration covers both chromatids and thereby comprises the sum of two independent observations. The integrated green and red fluorescence profiles that run from pter to qter of each chromosome are then used to calculate the green/red ratio profile for each chromosome. The set of these ratio profiles comprises the copy-number karyotype (Fig. 2).

Averaging of Profiles Collected From Several Metaphases

Random noise in the hybridization limits the ability to recognize copy number changes. Since CGH signals on chromosome homologues in each metaphase spread should be the same, we combine data from multiple metaphases to reduce the noise and thereby enhance the visibility of real copy number changes (Fig. 4). Data from at least four chromosomes from two or more high-quality metaphases are used and profiles of the mean ratio and $\pm$ 1 S.D. are calculated and displayed (Figs. 2 and 5).

While simple in concept, accurate implementation of profile averaging is difficult because the same chromosomes may be differently condensed, and the condensation may be non-uniform along the length of the chromosome. The former problem is easily solved by normalizing all ratio profiles to a standard length for that chromosome type. However, if the condensation is non-uniform, ratio changes may occur at slightly different locations along the medial axis of each chromosome. Thus, the combination of profiles from several chromo-

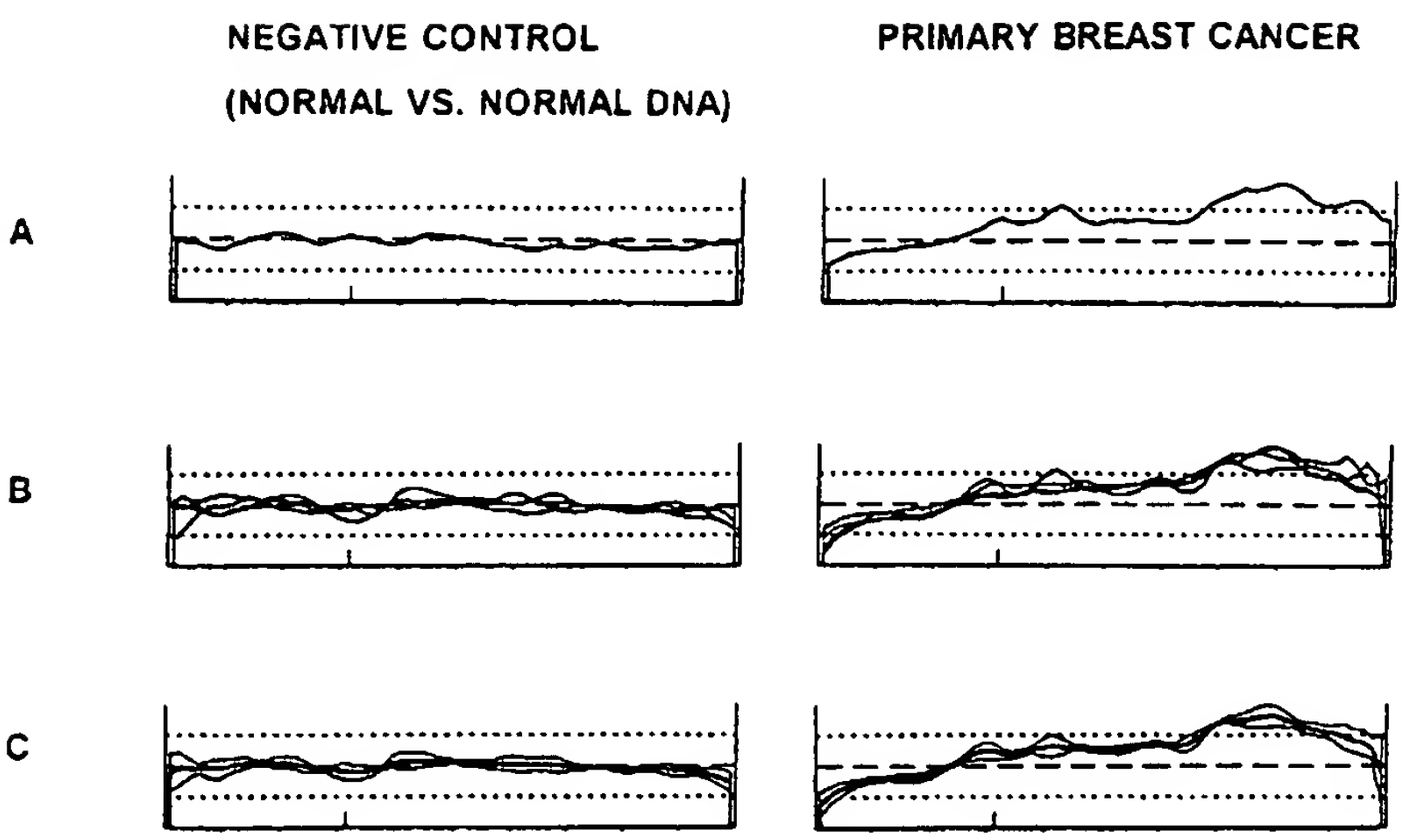

Figure 4. Combination of ratio data from many metaphases improves interpretation of genetic changes. Green to red ratio profiles from chromosome 8 are shown for a negative control experiment (left column, comparing two normal DNAs against one another) and for a primary breast cancer (right column). **A:** A single profile for chromosome 8 suggests that 8p is deleted and the distal 8q amplified in the tumor. The region in between remains questionable. **B:** Drawing profiles from 4 copies of chromosome 8 in a single plot and, **C:** calculating the mean ± 1 S.D. for the green to red ratio improves signal to noise ratio and the detection of small copy number aberrations. The proximal 8q shows a green to red ratio that is significantly elevated as compared to the control experiment, thereby verifying also that the proximal 8q is gained in the tumor.

somes may result in smearing of aberrations, especially those affecting small regions. Procedures based on the use of the chromosome banding pattern for multi-point registering along the entire chromosome length are currently under development.

Control Experiments and Quality Assurance

We include a comparison of two normal DNAs in each CGH experiment since it provides a negative control that is very helpful for interpretation of results from tumor specimens with a particular batch of slides and reagents. Only ratio changes that exceed the fluctuation seen in this control experiment are interpreted as evidence of real DNA gain or loss in tumor specimens. In a successful hybridization, this normal variation (± 1 S.D.) should not exceed ratios of 0.85 to 1.15 (Fig. 5). The telomeric, peri-centromeric or heterochromatic regions sometimes fall outside this range since the signal intensities are low. These regions are excluded from all analyses.

We also use a positive control, e.g., a cell line with known amplifications and deletions, in each experiment. The cell line is chosen to contain aberrations that are difficult to detect (e.g., low-level amplifications, deletions, etc.).

Guidelines for Interpretation of Ratio Data

While ratio imaging provides the most accurate and objective way to assess the "copy number karyotype," interpretation of the ratio profiles has yet to be standardized and automated. For the time being, we are using several criteria for interpreting the data:

1. Only high-quality hybridizations are evaluated. The consistency of the green to red ratio from one metaphase to another can be used to judge the quality of the hybridization (Fig. 2). If the standard deviation of the ratio profiles exceeds 0.15, we do not use the results.
2. Ratio profiles are compared with visual assessment of the digital images to determine that they are concordant. The breakpoints for gains and deletions are localized to chromosome bands by superimposing the coordinates of the three-color image with those of a single color DAPI image.
3. Ratio changes that clearly exceed the background ratio variation seen in the negative control experiment (Fig. 5) are interpreted as evidence of real copy number differences. The following regions are excluded from evaluation: centromeric and heterochromatic regions, p-arms of acrocentric chromosomes, and telomeric regions (see Limitations and Difficulties). Caution should also be exercised in interpreting ratio changes at 1p32-pter, 16p, 19, and 22 because of the high rate of apparently abnormal ratios in these regions in comparisons of two normal DNAs (see Limitations and Difficulties).

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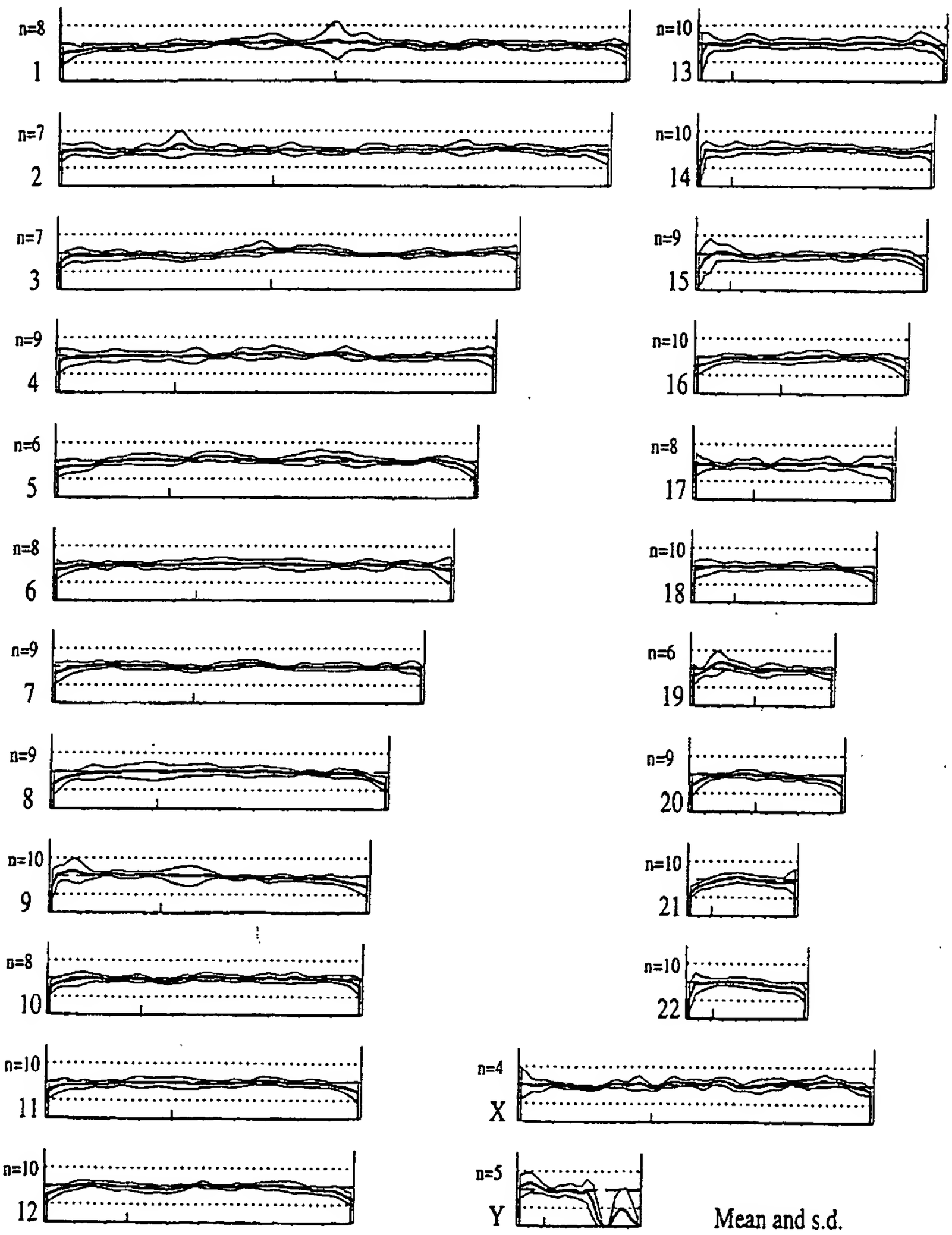

Figure 5. Green to red ratio profiles for a negative control experiment comparing two normal DNA specimens labeled in two different colors against one another. The mean ± 1 S.D. of the green to red ratio profiles are shown for each chromosome (n = 6–10) as described in the legend for Figure 2. No ratio changes are seen along any of the chromosomes.

VALIDATION, SENSITIVITY, AND MAPPING RESOLUTION OF CGH

Table 1 provides a summary of some of the experiments we have carried out to validate CGH. Most of these studies were based on genetically homogeneous established cell lines. Using five cell lines with varying numbers of X chromosomes in an otherwise diploid background, we showed that loss or gain of one copy of a chromosome was detectable and that there was a linear correlation between chromosome copy number (from 1 to 5) and the average green to red ratio (Kallioniemi et al., 1992). The quantitative linear relation between DNA sequence copy number and the green to red

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TABLE 1. A Summary of Main Experiments Carried Out to Validate CGH

1. Green to red ratio correlates linearly with the copy number when large regions of the genome, such as entire chromosomes, are studied (Kallioniemi et al., 1992).
2. High-level amplifications (> 5–7 ×) of known oncogenes (*MYC, ERBB2, BCL1*) are detectable in cancer cell lines (Kallioniemi et al., 1992; 1993a, 1994).
3. FISH studies of tumor interphase nuclei using anonymous cosmid probes that match the previously unknown amplified regions (such as 20q13 in breast cancer) found by CGH have indicated high-level (> 10-fold) amplifications (Kallioniemi et al., 1994).
4. Deletions spanning 10–20 Mb at known chromosomal locations are detectable in near-diploid cell lines (Kallioniemi et al., 1992).
5. CGH data on losses and gains of chromosomal regions are in 90–100% concordance with cytogenetic data from established near-diploid bladder cancer cell lines (Kallioniemi et al., unpublished data).
6. The concordance between losses of chromosomal regions by CGH and by RFLP analyses is about 75–85% (Kallioniemi et al., 1993b; Sakamoto et al., 1993).
7. Hybridization of two genetically normal DNAs, labeled in two different colors, against one another shows no color ratio differences (Fig. 5).

ratio is only obtained when the size of the region affected is large enough (>10–20 Mb changes have so far been demonstrated to follow this rule). However, qualitative detection of copy number aberrations affecting much smaller regions (a few hundred kilobases) is possible if the DNA sequences show high-level amplification. We estimate that the total amount of amplified DNA (amplicon size times level of amplification) has to be at least 2 Mb for it to become detectable by CGH (Piper et al., 1994).

Detection of 10–20 Mb or larger deletions is possible in cell lines with a diploid modal chromosome number (Kallioniemi et al., 1992). We have not fully examined the smallest size of deletions detectable, but expect it to be around 3–5 Mb which is the smallest distance that can be adequately discriminated by FISH in the metaphase analysis of two probes. The sensitivity for deletion detection is also critically dependent on ploidy level. For example, it is more difficult to detect a loss of one chromosome homologue in a tetraploid cell line than in a diploid one. In the tetraploid cell line, the green to red ratio is expected to change only by 25% (from four to three copies) as compared to 50% in the diploid cell line (from two to one copy).

A few experiments have been done to determine the sensitivity of CGH in the analysis of primary tumors. High-level amplifications of known oncogenes are detectable in primary tumors, as are amplifications involving many previously unrecognized chromosomal loci (Kallioniemi et al., 1994). Deletions also appear to be detected with high probability. A 75–85% concordance has been found between the presence of chromosome deletions by CGH and loss of heterozygosity (LOH) by RFLP in breast (Kallioniemi et al., 1993b) and ovarian (Sakamoto et al., 1993) cancer. Part of the discordant cases may be explained by the fact that LOH may be caused by mechanisms that do not involve physical losses of DNA, such as mitotic recombination, gene conversion, and non-disjunctional loss of a chromosome followed by duplication of the other copy.

Experience from physical mapping of cosmids to metaphase chromosomes by FISH suggests that an accuracy of 3–5 Mb may be achieved by CGH in the assignment of ratio changes to human chromosomes. These limits are determined by the packing of DNA in the metaphase chromosomes and the effects of denaturation on chromosome morphology. If chromosome bands are used as a reference for mapping, the number of bands obtained with the staining method used sets the limit. After hybridization, DAPI banded chromosomes provide 10–20 Mb resolution at best. Better banding methods will improve accuracy for band assignments.

LIMITATIONS AND DIFFICULTIES

Balanced Genetic Aberrations

CGH only detects genetic aberrations that involve loss or gain of DNA sequences. Thus, balanced translocations or inversions are not detectable, nor are point mutations and small intragenic rearrangements.

Ploidy Changes

CGH only detects DNA sequence copy number changes relative to the average copy number in the entire tumor specimen. For example, CGH cannot distinguish diploid tumors from true triploid or tetraploid cases as all these tumors would show no

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differences in the green red ratio between or within the chromosomes. The relative green to red ratios can be transformed to indicate actual copy numbers if the absolute copy number for several loci are independently determined (e.g., using FISH and locus-specific probes to analyze the tumor cells directly) or if the ploidy is determined by, e.g., DNA content analyses (Kallioniemi et al., 1992).

Peri-Centromeric and Heterochromatic Regions

Peri-centromeric and heterochromatic repeat regions cannot be reliably evaluated by CGH as they are being blocked to various extents by the unlabeled Cot-1 DNA in the hybridization. These DNA sequences are highly polymorphic in copy number between individuals. Thus, ratio changes at or near these regions should be interpreted cautiously, especially when the test and reference DNA samples come from different individuals.

Telomeric Regions

The green and red fluorescence intensities gradually decrease at the chromosome telomeres. When the absolute intensities start to approach the background fluorescence, unreliable ratio changes may appear. Caution should therefore be exercised in the interpretation of ratio changes at chromosome telomeres.

Normal Cell Contamination

Similar to other methods based on isolated DNA, CGH requires that the tumor specimens are relatively free of surrounding normal tissues that dilute the green to red ratio changes (Fig. 3). This is a significant problem in some tumor types, such as prostate cancer, where malignant cells are often surrounded by large numbers of nonmalignant hyperplastic cells. If the normal tissue contribution is greater than 50% of the total DNA content, reliable detection of ratio changes becomes increasingly difficult.

Intratumor Genetic Heterogeneity

CGH detects the average copy number of sequences in all cells included in the specimen. Thus, those aberrations that are homogeneously present (clonal) in the tumor cells are more readily detected. In most cases, this is an advantage, as the clonal changes are likely to represent the early and most important ones. However, in bi- or multiclonal tumors, the different genetic aberrations present in the individual clones may sometimes balance one another or exist at too low a frequency to be detected.

Systematic Locus-Specific Ratio Fluctuations

Recent evidence indicates that in control experiments with a normal biotin-labeled and a normal digoxigenin-labeled DNA sample (with avidin-FITC and anti-digoxigenin rhodamine detection), the green to red fluorescence ratios at 1p32-pter, 16p, 19, and 22 may occasionally be distinctly below the average ratio. This would therefore lead to a false positive interpretation of a deletion. Other chromosomal regions do not seem to be affected. False positive gains of DNA sequences have not been seen at any locus using these nucleotide labels. Preliminary evidence indicates that these problems are due to differential hybridization properties of digoxigenin and biotin-labeled probes. There also seems to be a lack of correlation between RFLP and CGH in detecting deletions on chromosome 19 (Sakamoto et al., 1993). Further studies focusing on these regions are currently under way and should clarify the extent of the problem. For the time being, we exclude these regions from the analysis of deletions. It may be possible to circumvent this methodological problem by reversing the labels in a control experiment, e.g., tumor labeled with digoxigenin (detected with rhodamine) and normal DNA with biotin (detected with FITC). Only ratio changes that invert with the inversion of the labels are interpreted as real. The use of directly fluorochrome-conjugated nucleotide labels may also help to minimize these locus-specific hybridization problems, but extensive testing of each labeling combination is required.

CONCLUSIONS

CGH technology at its current state of development is primarily a research tool. Although the technology has been refined and many interesting applications can be envisioned, further research and technical development is required before clinical application is routine.

CGH supplements, not replaces current technologies. In providing genome-scale overviews of DNA sequence copy number changes from common solid tumors, CGH provides access to practically every clinical specimen regardless of mitotic activity, as well as allows detailed analysis and interpretation of even the most chaotic and complex karyotypes. It also maps the origins of amplified and deleted DNA sequences on normal chromosomes, thereby highlighting locations of important genes. However, sensitivity to detect translocations and inversions as well as the ability to distin-

guish different subclones and thereby trace the clonal evolution of tumors remain the strengths of conventional karyotyping.

CGH is also complementary to specific molecular genetic investigations (such as Southern, RFLP, their PCR-based variations as well as FISH) of specific loci by providing genome-scale information that may help to focus detailed molecular studies to regions that are most likely to be important. For example, CGH analyses have indicated that amplified sequences originating from 20q13 are common in breast cancer (Kallioniemi et al., 1994). We have recently used cosmid probes for 20q13 in interphase FISH analysis of primary tumor specimens to validate high level amplifications of this locus and to narrow down the minimal common region of amplification to a small segment of this band so that positional cloning efforts to identify this potential new breast cancer oncogene can be initiated (manuscript in preparation).

It is likely that improvement of CGH methodology will continue. This will lead to improved sensitivity to small aberrations and increased robustness and minimal variability from one experiment to another. Several modifications of the CGH technique, such as comparisons of two abnormal genomes (e.g., primary vs. metastasis), the use of universal-primed PCR-amplified DNA from microdissected tumor regions or even single cells, hybridizations comparing RNA instead of DNA, or comparative genomic hybridizations to artificial targets (such as high-density filters from genomic libraries) are also likely to be developed.

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APPENDIX. Laboratory Protocol for Comparative Genomic Hybridization

Nick translation of DNA samples for comparative genomic hybridization

In a microcentrifuge tube, mix:
- 1 μg tumor DNA in 38 μl double-distilled water
- 5 μl 10 × A4 mixture[a]
- 1 μl (~1 nmol) FITC-12-dUTP (DuPont, Boston, MA)[b]
- 5 μl enzyme mixture containing DNA polymerase I (~2 U) and DNAse I (~200 pg) (Gibco BRL, Gaithersburg, MD)
- 1 μl (~10 U) DNA polymerase I (Promega, Madison, WI)

Incubate 45–60 min at 15°C
Stop reaction by a 10 min incubation at 70°C

Run a double-stranded 1% agarose gel to check the probe fragment distribution. Size range should be about 500–2,000 bp. If the probe fragments are longer, increase the incubation time or the amount of enzyme mixture and if shorter, decrease the incubation time or the amount of enzyme mixture.

Comparative genomic hybridization

Preparation of target metaphase slides:
- Mark the area of the slide containing metaphases with a diamond pen
- Denature normal lymphocyte metaphase slides for 3 min at 74°C in a denaturation solution (70% formamide, 2 × SSC, pH 7, in a coplin jar)
- Dehydrate in a sequence of 70, 85, and 100% ethanol, 2 min each
- Incubate slides in a proteinase K solution (0.1 μg/ml in 20 mM Tris-HCl/2 mM CaCl, pH 7.5) for 7.5 min at room temperature
- Dehydrate as above

Preparation of hybridization mixture:
- Mix in a microcentrifuge tube:
  - 10 μl labeled tumor DNA from the nick translation reaction (200 ng)
  - 10 μl labeled normal DNA from the nick translation reaction (200 ng)
  - 10 μl Cot-1 DNA (10 μg, from Gibco-BRL)
  - 3 μl 3 M sodium acetate
  - 66 μl 100% ethanol
- Precipitate by centrifuging for 30 min in a microcentrifuge (~14,000 rpm)
- Decant and let dry
- Dissolve the pellet in 10 μl hybridization buffer (50% formamide, 10% dextran sulfate, 2 × SSC, pH 7)
- Denature 5 min at 70°C immediately before use

Hybridization and washing:
- Pipet 10 μl of hybridization mixture on slide, apply an 18 × 18 mm coverslip and seal with rubber cement
- Hybridize for two days at 37°C in a moist chamber
- Remove coverslip and wash slides three times in a washing solution (50% formamide, 2 × SSC, pH 7) at 45°C for 10 min each
- Wash twice in 2 × SSC at 45°C and once at room temperature, 10 min each
- Wash once in PN (a mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 (pH 8), and 0.1% Nonidet P-40) and once in distilled water (both 10 min at room temperature)
- Air-dry slides and mount in an anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA) containing 0.1–0.2 μM DAPI

[a]10 × A4 is 0.2 mM dATP, dCTP, dGTP in 500 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 100 mM β-mercaptoethanol, 100 μg/ml BSA (nuclease free).
[b]For labeling of normal reference DNA, FITC-12-dUTP is substituted with TexasRed-5-dUTP (DuPont).

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